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Profiling Neurosteroids in Cerebrospinal Fluids and Plasma by Gas Chromatography/Electron Capture Negative Chemical Ionization Mass Spectrometry

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A quantitative method for the determination of allopregnanolone (5α,3α-THP) and related neurosteroids in CSF and plasma was established using gas chromatography/electron capture negative chemical ionization mass spectrometry (GC/ECNCI/MS). Neurosteroids were converted to carboxymethoxime, pentafluorobenzyl and trimethylsilyl derivatives and detected as intense $(M-181)^-$ fragment ions generated under the negative ion chemical ionization process. The response curves constructed using d_4-dihydrotestosterone (DHT) and d_4-5α,3α-THP as internal standards showed linearity in the concentration range of 10–1000 pg/ml. The variation of response ratios determined against internal standards over a 2-month period was less than 10%. Instrumental detection limits for most neurosteroids were in the low picogram range with the exception of progesterone and dihydroprogesterone (DHP) which were detected with approximately 10 times less sensitivity in comparison to other steroids. In conjunction with solid-phase extraction, this method allowed the quantification of at least four neurosteroids, including androsterone, testosterone, 5α,3α-THP, and pregnenolone in 1–2 ml of human cerebrospinal fluid (CSF). While the level of 5α,3α-THP in human CSF was comparable to that in the human plasma, other steroid levels were significantly lower. Although individual CSF and plasma samples showed widely varying neurosteroid levels, species specificity appeared to exist. The levels of 5α,3α-THP and pregnenolone in human CSF were higher than those of monkey CSF where these steroids were often not detected with our current detection limit. In comparison to human plasma, rat plasma samples contained considerably lower levels of androsterone and pregnenolone. Among THP stereoisomers, 5β,3α-THP and 5α,3β-THP were observed only in human plasma, while 5β,3β-THP was detected only in rat plasma.

Key Words: **neurosteroids; allopregnanolone; pregnanolone; isopregnanolone; epipregnanolone; gas chromatography/mass spectrometry; electron capture negative ion chemical ionization; cerebrospinal fluids; plasma.**

Neurosteroids are pregnane and androstane steroids that are synthesized *de novo* in the central nervous system (1, 2). Increasing evidence has shown that neurosteroids play a central role in many neuronal processes, particularly in the modulation of $GABA_A$ receptor-mediated functions (2–4). Among these, 5α,3α-tetrahydroprogesterone (5α,3α-THP[2] or allopregnanolone) and 5α,3α-tetrahydrodeoxycorticosterone (5α,3α-THDOC or allo-THDOC) are potent agonists of the $GABA_A$ receptor (5–7). Interaction of 5α,3α-THP and 5α,3α-THDOC with $GABA_A$ receptor has also been shown to enhance the hypnotic, anxielytic, and anesthetic effects of $GABA_A$ (8–10). On the other hand, pregnenolone (PREG) sulfate and dehydroepiandrosterone (DHEA) sulfate, the conjugated forms of PREG and DHEA, have been indicated to be $GABA_A$ receptor antagonists (11, 12).

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[2] Abbreviations used: THP, tetrahydroprogesterone; 5α,3α-THP, allopregnanolone; 5β,3α-THP, pregnanolone; 5α,3β-THP, isopregnanolone; 5β,3β-THP, epipregnanolone; DHT, dihydrotestosterone; PREG, pregnenolone; DHP, dihydroprogesterone (5α-pregnan-3,20-dione); DHEA, dehydroepiandrosterone; THDOC, tetrahydrodeoxycorticosterone; CSF, cerebrospinal fluid; GC/MS, gas chromatography/mass spectrometry; ECNCI, electron capture negative chemical ionization; SPE, solid-phase extraction; LC/MS, liquid chromatography/mass spectrometry.

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3β-Hydroxy-Δ5-neurosteroids, such as PREG and DHEA, are metabolic intermediates between cholesterol and their metabolites, progesterone and testosterone (2). Allopregnanolone (5α,3α-THP) is a product of further metabolism of progesterone and its biosynthesis in the brain has been shown to be manipulated through various mechanisms (13–15). Since these compounds are metabolically associated (2), changes in the endogenous levels of specific neurosteroids may reflect *in vivo* regulation of their biosynthesis and metabolism, which in turn may provide insights to their physiological implications.

Analytical procedures currently available for the quantitative analysis of steroidal compounds include radioimmunoassay (16, 17), gas chromatography/mass spectrometry (GC/MS) (18–20), high-performance liquid chromatography (21), and liquid chromatography/mass spectrometry (22–24). While these techniques have been successfully employed in the determination of selected steroids in given tissues and/or biological fluids, quantitative methods for the analysis of neurosteroids, especially 5α,3α-THP in a reasonable volume of cerebrospinal fluids (CSF) have been difficult due to low CSF concentrations of these compounds. In the present study, we established a method using GC/EC-NCI/MS to profile and quantify $GABA_A$ active neurosteroids in CSF and plasma samples. This method demonstrates the capability of simultaneous analysis of several neurosteroids present in CSF and plasma, thus providing an invaluable tool for clinical investigations on psychiatric disorders that are believed to be associated with specific neurosteroid profiles.

MATERIALS AND METHODS

Chemicals

Steroid standards (>98% purity) were purchased from Steraloids, Inc. (Wilton, NH) and from Sigma Chemical Co. (St. Louis, MO). Deuterium-labeled standards (progesterone-1,2,6,7-d_4, 5α,3α-THP-17,21,21,21-d_4, 5α,3α-THDOC-17,21,21-d_3, 5α-DHP-1,2,4,5,6,7-d_6, and 5α-DHT-1,2,4,5-d_4) were obtained from Cambridge Isotope Laboratories (Andover, MA). Derivatizing agents including carboxymethoxylamine hemihydrochloride and pyridine were purchased from Aldrich, pentafluorobenzyl bromide and diisopropylethylamine were from Pierce, and bis(trimethylsilyl) trifluoroacetamide was from Alltech. Organic solvents were HPLC grade from Burdick & Jackson (Muskegon, MI), and deionized water was obtained using a Milli-Q reagent water purification system (Millipore, Bedford, MA).

Experimental Procedures

CSF and plasma collection. Human CSF samples from normal volunteers (male 33–72 years old) were drawn by the lumbar puncture after an overnight fast as previously described (25). Cisternal monkey CSF samples were collected as described earlier (26). Human blood samples were collected in heparin after an overnight fast. Rat blood samples were collected in heparin by decapitation of male Sprague–Dawley rats (250–300 g body wt). Plasma samples were prepared by centrifugation at 1000*g*.

Extraction. Neurosteroids in both CSF and plasma samples were isolated by solid-phase extraction (SPE). In short, 1–2 ml CSF or 300 μl plasma samples was spiked with 140–280 pg of deuterium-labeled standards and applied onto 100 mg C18 SPE columns (Varian Sample Preparation Products, Harbor City, CA) which had been preconditioned with 4 ml methanol and 4 ml deionized water. After washing the loaded columns with 4 ml deionized water, neurosteroids were eluted with 2 ml methanol. For the evaluation of reproducibility and for the generation of calibration curves, 1 ml saline solution was used instead of CSF or plasma. The saline solutions containing various amounts of steroid standards were spiked with 70 pg each of deuterium-labeled standards and the SPE extraction procedure was performed. Since the response of deuterium-labeled internal standards was linear in the range of 1–1000 pg, standard curves generated with 70 pg of internal standards were interpolated for the quantitation of steroids in plasma or CSF samples spiked with 140–280 pg of internal standards.

Derivatization. Neurosteroids were derivatized according to the method of Hubbard *et al.* (19) with slight modifications. Briefly, the neurosteroids were reacted with various reagents in the following sequential order: 50 μl of 0.2% carboxymethoxylamine hemihydrochloride in pyridine at 60°C for 45 min, 100 μl of 1.25% pentafluorobenzyl bromide and 2.5% diisopropylethylamine in acetonitrile at 45°C for 20 min, and 100 μl of 50% bis(trimethylsilyl)trifluoroacetamide in acetonitrile at 45°C for 30 min. After each step the reaction mixture was dried down completely under nitrogen stream prior to proceeding to the next step. Unless specified, the fully derivatized steroid samples were dissolved in 5 μl of hexane and 4 μl was injected into GC/MS using the splitless injection technique.

Instrumentation. GC/MS analysis was carried out using an Hewlett–Packard (HP) 5989A mass spectrometer coupled to an HP 5890 gas chromatograph. A thin-film capillary column (15 m × 0.25 mm, 0.05 μm film thickness; Quadrex Corp., New Haven, CT) was used to resolve the derivatives of various neurosteroids. The oven temperature was raised from 150 to 230°C at 30°C/min, 230 to 250°C at 1°C/min, and 250 to 320°C at 30°C/min. The injector and transfer-line temperatures were maintained at 300 and 310°C, respectively. Derivatized steroids were first analyzed quali-

tatively by full scanning in the mass range of 250–950. For quantification, the mass spectrometer was operated in the selected ion monitoring mode. The temperatures of mass spectrometer source and quadrupole were set at 200 and 100°C, respectively.

Construction of calibration curves. Neurosteroids in the range of 10 to 1000 pg in 1 ml saline were passed through a solid-phase extraction column in the presence of 70 pg d_4-DHT and d_4-5α,3α-THP, and the eluted neurosteroids were derivatized as described above. Calibration curves were generated for 10–1000 pg of starting material using the GC/MS response ratios calculated against the responses of deuterium-labeled internal standards. Each data point represented an average of three different determinations. The responses of testosterone, dihydrotestosterone, and androsterone were calibrated using d_4-DHT, and for THP stereoisomers and pregnenolone d_4-5α,3α-THP was used as an internal standard. The detection limit for each neurosteroid was estimated according to a signal to noise ratio of 3 to 1. Standard mixtures containing 5 to 50 pg of steroids were extracted and derivatized and one-fifth was injected to evaluate the detection limit.

Reproducibility. A stock solution of standard neurosteroid mixture spiked with internal standards was prepared and analyzed periodically to evaluate reproducibility. The GC/MS response ratios for each steroid were calculated against the corresponding internal standards. The variations of the response ratios were monitored for individual neurosteroids over a period of 2 months.

RESULTS AND DISCUSSION

GC/MS Analysis of Neurosteroids

Neurosteroids targeted in the present study were structurally related albeit differ in the numbers of carbonyl and hydroxyl groups. These polar groups were derivatized to enhance volatility as well as detection sensitivity. To detect picogram levels of allopregnanolone and related steroids in CSF, the electron capturing ability of pentafluorobenzyl derivatives was exploited as described previously for corticosteroids (19). The carbonyl group was first oximated via carboxymethoxymation to introduce a carboxyl group and pentafluorobenzyl esterification was performed subsequently to include a strong electron capturing moiety. The hydroxyl group was converted to trimethylsilyl ether, a common step to enhance the volatility of hydroxylated compounds. In the negative chemical ionization mode, the predominant ion observed for each individual neurosteroid was $(M\text{-}181)^-$ due to loss of the pentafluorobenzyl group ($-CH_2C_6F_5$). Figure 1 shows typical mass spectra of DHT (*m/z* 434) and d_4-DHT (*m/z* 438) as well as allopregnanolone (*m/z* 462) and d_4-allopregnanolone (*m/z* 466). The generation of these characteristic and intense ions along with retention behavior provided the basis for identification and quantitation of neurosteroids in biological matrices. The isotopic purity of d_4-DHT was 92.7% and contained considerable proportion of d_3 (25%) and d_2 isotopes (2%) based on the evaluation by both GC/MS and electrospray LC/MS (data not shown) while other internal standards showed isotopic purity greater than 98%. None of the internal standards contained any detectable d_0 component and therefore did not interfere with the analysis of endogenous steroids.

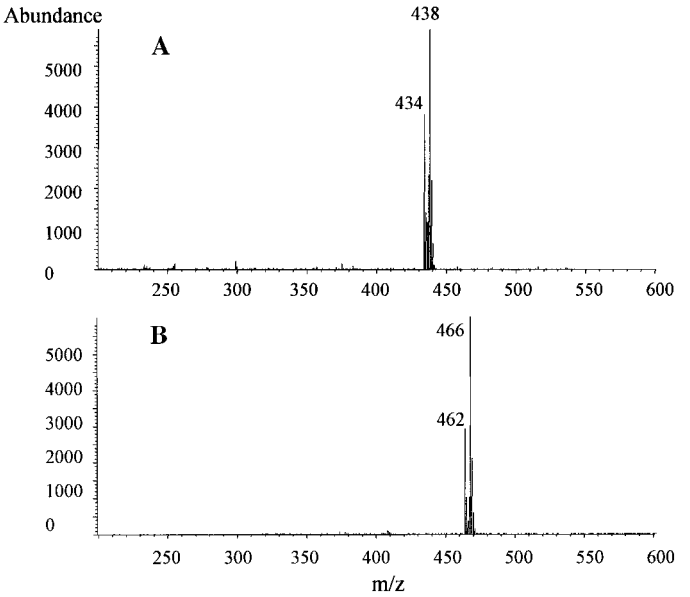

FIG. 1. Full scan mass spectra obtained by GC/ECNCI/MS for (A) d_0- and d_4-dihydrotestosterone (*m/z* 434 and 438) and (B) d_0- and d_4-allopregnanolone (*m/z* 462 and 466). Two nanograms each of d_0 and 2.8 ng each of d_4 standards was analyzed.

The carboxymethoxymation and the subsequent pentafluorobenzyl esterification added considerable mass to the parent molecules. A regular nonpolar 30-m × 0.25-mm capillary GC column with a film thickness of 0.25 μm required extended running time, thus limiting the number of analyses that could be performed per day. The thin film capillary GC column (0.05 μm) described by Hubbard *et al.* (19) allowed a relatively quick elution for various derivatized neurosteroids while providing adequate resolution of these compounds. Figure 2 shows a typical selected ion profile of neurosteroids (Fig. 2A) and deuterium-labeled internal standards (Fig. 2B) obtained in a single GC run. The oximated carbonyls of the fully derivatized neurosteroids yielded *syn*- and *anti*-isomers, thus generating doublets in some cases when chromatographically separated as shown for testosterone, DHT, progesterone and DHP. Although syn- and anti-isomers did not form in a consistent proportion, quantitation based on both

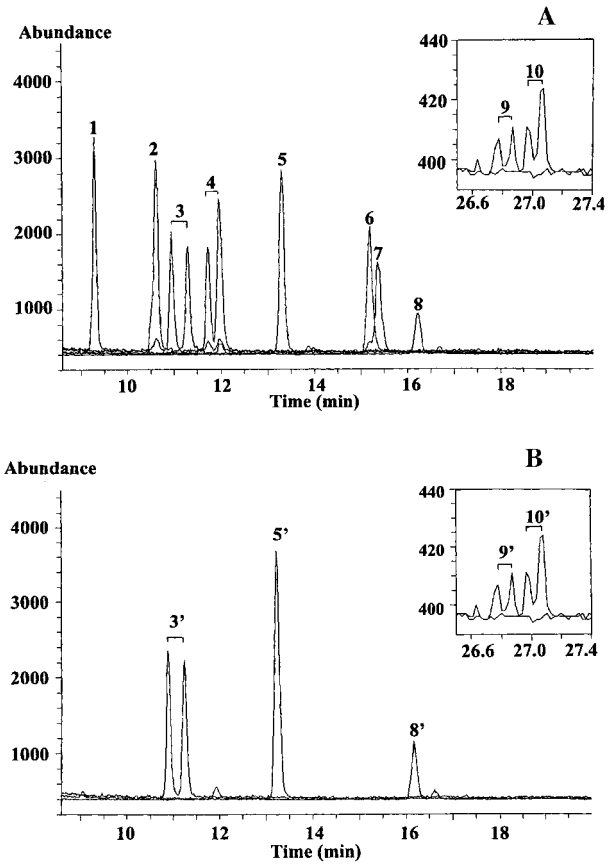

FIG. 2. Separation and detection of neurosteroids by GC/ECNCI/MS. Five hundred picograms of d_0 and 700 pg of d_4 steroids were derivatized, one-tenth of which was injected. (A) GC/ECNCI/MS selected ion chromatograms of d_0 steroids including (1) androsterone (*m/z* 434), (2) dehydroepiandrosterone (*m/z* 432), (3) dihydrotestosterone (*m/z* 434), (4) testosterone (*m/z* 432), (5) allopregnanolone (*m/z* 462), (6) pregnenolone (*m/z* 460), (7) isopregnanolone (*m/z* 462), (8) allotetrahydrodeoxycorticosterone (*m/z* 550), (9) dihydroprogesterone (*m/z* 641), and (10) progesterone(*m/z* 639). (B) GC/ECNCI/MS selected ion chromatograms of deuterium-labeled internal standards including (3′) d_4-dihydrotestosterone (*m/z* 438), (5′) d_4-allo-pregnanolone (*m/z* 466), (8′) d_3-allotetrahydrodeoxycorticosterone (*m/z* 553), (9′) d_6-dihydroprogesterone(*m/z* 647), and (10′) d_4-progesterone(*m/z* 643).

peaks produced consistent results. It is worthwhile to note that derivatives of neurosteroids that contain two carbonyl groups, such as progesterone and dihydroprogesterone, required longer elution time (Fig. 2, inset). Inevitably, the sensitivity for these compounds was significantly reduced. Under the chromatographic conditions employed, androsterone and dehydroepiandrosterone were completely separated from their corresponding positional isomers, DHT and testosterone, respectively.

Sample Preparation

To achieve a sensitive and reproducible GC/MS analysis of neurosteroids in CSF samples, a proper sample preparation procedure to eliminate undesired interference was required. Both liquid–liquid extraction with ethyl acetate and solid-phase extraction (SPE) with C18 columns were evaluated in the present study. While ethyl acetate extraction was convenient and less time-consuming, it failed to provide satisfactory results in reducing background interference. On the other hand, SPE with 100 mg (or 1 ml) C18 columns (Varian Sample Preparation Products) consistently yielded greater than 80% recovery for most neurosteroids tested (Table 1). It is noteworthy that the recovery suffered greatly when 500-mg C18 SPE columns (or 3 ml) were used, probably due to increased irreversible adsorption of neurosteroids on the surface of column packing material. The detection of 5α,3α-THDOC (*m/z* 550) was interfered with by the background peaks at *m/z* 550 appearing around its GC retention time after SPE. The overall recovery of DHT and 5α,3α-THP from biological matrices was also tested using 140 pg each of deuterium-labeled compounds. Greater than 90 or 70% of these standards was recovered from CSF or plasma, respectively.

Calibration Curves and Detection Limit

Since deuterium-labeled standards were not available for each neurosteroid, various neurosteroids were determined using d_4-DHT and d_4-5α,3α-THP as internal standards. The responses of androsterone and dihydrotestosterone (*m/z* 434) and dehydroepiandrosterone (DHEA) and testosterone (*m/z* 432) were calibrated using d_4-dihydrotestosterone (DHT). For stereoisomers of tetrahydroprogesterone including pregnanolone and allo-, epi-, and isopregnanolone (*m/z* 462) as well as pregnenolone (*m/z* 460), d_4-allopregnanolone (5α,3α-THP) was used as an internal stan-

TABLE 1

Recovery of Steroid Standards during the SPE Procedure

Neurosteroids	Recovery (%)
Androsterone	78.2 ± 1.5
Dihydrotestosterone	99.5 ± 1.1
Testosterone	89.5 ± 2.1
Allopregnanolone	94.2 ± 5.9
Isopregnanolone	88.6 ± 4.7
Pregnenolone	92.6 ± 3.0

Note. A standard mixture containing 100 pg each of steroids was applied onto SPE columns in 1 ml of saline solution. After solid-phase extraction 70 pg each of deuterium-labled internal standards was added. A separate aliquot of standard mixture was spiked with 70 pg each of internal standards, directly derivatized, and used as a reference. The recovery was calculated by comparing the GC/MS response ratios calculated against deuterium-labeled internal standards with those of the reference. Figures represent the mean ± standard deviation (n = 6).

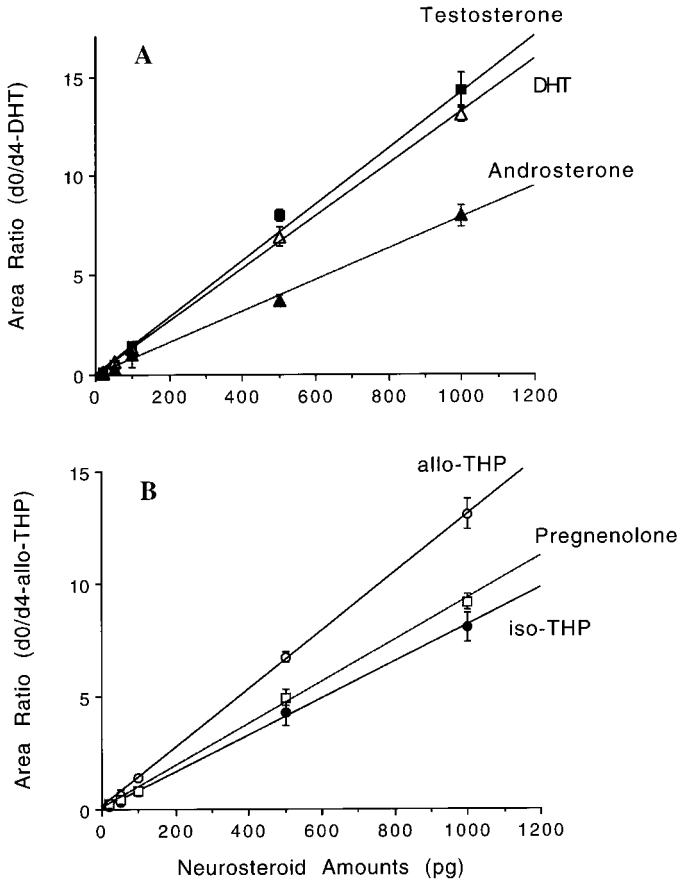

FIG. 3. Calibration curves for various steroids generated in the presence of 70 pg deuterium-labeled internal standards. Steroids (10–1000 pg) and internal standards were applied onto SPE column in saline solution, collected, and derivatized for GC/ECNCI/MS analysis. (A) Testosterone (closed square, $R^2 = 0.996$), dihydrotestosterone (open triangle, $R^2 = 0.999$), and androsterone (closed triangle, $R^2 = 0.998$) were calibrated against d_4-dihydrotestosterone. (B) allopregnanolone (open circle, $R^2 = 1.000$), pregnenolone (open square, $R^2 = 0.998$), and iso-pregnanolone (closed circle, $R^2 = 0.999$) were calibrated against d_4-allopregnanolone.

dard. As shown in Fig. 3, the response was linear in the range of 10 to 1000 pg of starting material for all the neurosteroids tested. However, the slopes of these calibration curves were different from one another, suggesting that instrumental responses, recoveries from solid-phase extraction, and/or derivatization yield may vary for each individual neurosteroid. Typical detection limits for most of the neurosteroid standards tested in saline starting with the solid-phase extraction procedure were in the range of approximately 1–5 pg of starting material, assuming a signal to noise ratio of 3 to 1. The detection limit in the presence of CSF or plasma matrices was generally two to three times higher. Although sensitivity of the technique depended largely on the cleanness of sample preparations, the sensitivity and quantitative ranges of the present method were suitable for the determination of neurosteroids in 1–2 ml of human and monkey CSF with the exception of progesterone and DHP. As discussed above, the presence of two carbonyl groups in progesterone and DHP yielded much heavier derivatives that required longer elution time, resulting in the broader chromatographic peaks. As a consequence, the detection limit for these compounds was approximately 10 times higher than that of the other neurosteroids.

Reproducibility

To test the reliability of the technique, variations in response factors of seven neurosteroids were monitored over a 2-month period. Individual steroids showed less than 10% variation, indicating that d_4-DHT and d_4-5α,3α-THP can be reliably used as internal standards to determine most neurosteroids tested (Table 2). The response ratios of DHEA calculated against d_4-DHT were not stable over a long time period even though the analyte responded with good sensitivity. Selection of a proper internal standard may improve reproducibility of the technique for the analysis of DHEA.

Neurosteroids in Cerebrospinal Fluids and Plasma

Neurosteroids in CSF samples were identified according to characteristic $(M\text{-}181)^-$ ions in conjunction with their GC retention times. Figures 4 and 5 show the representative GC/ECNCI/MS ion chromatograms of neurosteroids present in male human and monkey CSF samples, respectively. Several neurosteroids could be identified from CSF samples and their levels were determined using calibration curves constructed as described above. As shown in Table 3, androsterone, testosterone, allopregnanolone, and pregnenolone were

TABLE 2

Reproducibility of Neurosteroid Analysis

Neurosteroids	Deviation (%) from the mean
Androsterone	8.7
Dihydrotestosterone	4.7
Testosterone	10.1
Allopregnanolone	4.6
Isopregnanolone	6.9
Pregnenolone	9.2
Dehydroepiandrosterone	35.0

Note. Periodically ($n = 4$), an aliquot of a stock solution containing 100 pg each of neurosteroids was spiked with 140 pg each of deuterium-labeled internal standards, applied onto a SPE column in saline solution, and processed as described under Materials and Methods. The GC/MS response ratios (d_0/d_4) for androsterone, dihydrotestosterone, and testosterone were obtained using d_4-dihydrotestosterone and those for allopregnanolone, isopregnanolone, and pregnenolone were obtained using d_4-allopregnanolone. The variations of the response ratios were monitored over a 2-month period to evaluate reproducibility.

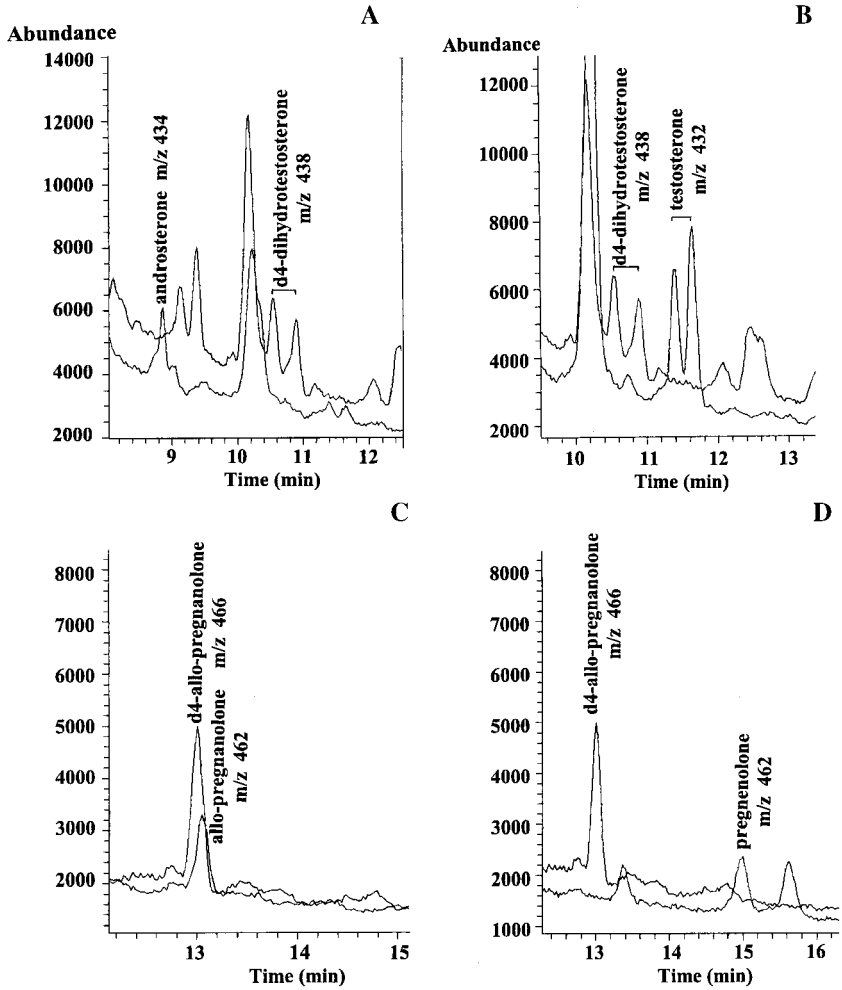

FIG. 4. GC/ECNCI/MS selected ion chromatograms of neurosteroids in a male human CSF sample. Typically, 1–2 ml of CSF samples was spiked with 140 pg each of d_4-dihydrotestosterone and d_4-allopregnanolone prior to SPE and derivatization processes. Androsterone (*m/z* 434, 52.8 pg/ml) (A) and testosterone (*m/z* 432, 158.3 pg/ml) (B) were quantitated using d_4-dihydrotestosterone. Allopregnanolone (*m/z* 462, 44.1 pg/ml) (C) and pregnenolone (*m/z* 460, 52.8 pg/ml) (D) were quantitated using d_4-allopregnanolone.

detected in male human CSF samples. Male monkey CSF samples also contained androsterone and testosterone but contained considerably lower levels of allopregnanolone and pregnenolone in comparison to human CSF.

Human plasma contained androsterone, DHT, testosterone, pregnanlone, allo-, and isopregnanolone as well as pregnenolone (Table 4). Rat plasma displayed a similar profile except for the fact that rat plasma is devoid of DHT and contained considerably less pregnenolone and androsterone. Under the present analysis conditions, stereoisomers of THP such as pregnanolone, allopregnanolone, and isopregnanolone were reasonably well separated, although epipregnanolone was only partially resolved from allopregnanolone (Fig. 6A). This allowed us to observe distinctive differences in the distribution of THP stereoisomers in rat and human plasma samples (Fig. 6 and Table 4). As shown in Figs. 6B and 6C, pregnanolone and isopregnanolone were not present in rat plasma while they were clearly detected in human plasma. Conversely, the presence of epipregnanolone appears to be unique in rat plasma. Among THP stereoisoforms, only 5α,3α (allopregnanolone) and 5β,3α forms (pregnanolone) are positive modulators of $GABA_A$ receptors (27). Therefore, the differences in their levels in CSF and plasma may reflect species specificity in the biosynthesis and metabolism of these steroids as well as their involvement in the modulation of $GABA_A$ function.

SUMMARY

Increasing evidence has shown that neurosteroids play important roles in neuronal functions (2, 27). However, quantitative and regulatory aspects of these

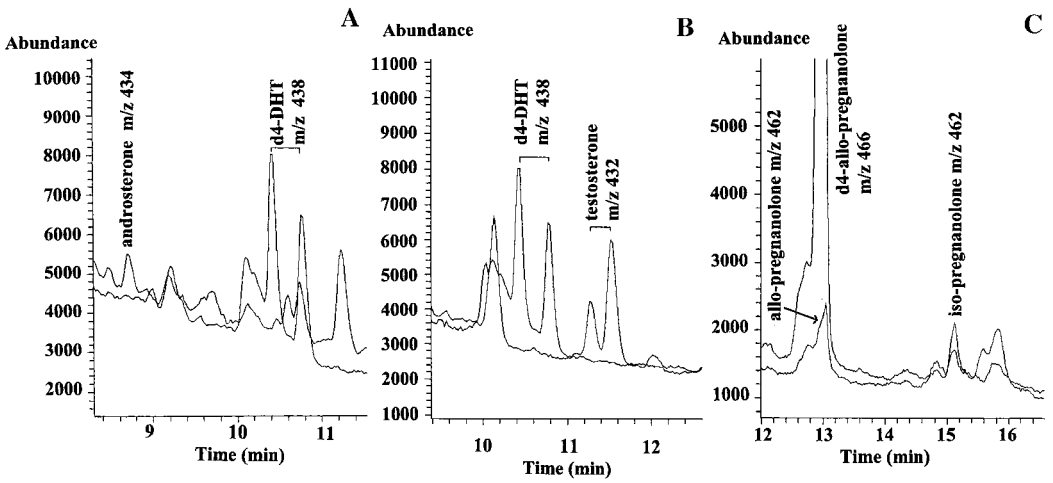

FIG. 5. GC/ECNCI/MS selected ion chromatograms of neurosteroids in a male monkey CSF sample. Androsterone (*m/z* 434, 24.7 pg/ml) (A) and testosterone (*m/z* 432, 73.7 pg/ml) (B) were quantified using 140 pg of d_4-dihydrotestosterone. Allopregnanolone (*m/z* 462, 6.3 pg/ml) and isopregnanolone (*m/z* 462, 16.7 pg/ml)) (C) were quantified against 140 pg of d_4-allopregnanolone.

compounds in the CNS are not yet fully understood. In the present study, a GC/MS method with electron capture negative ion chemical ionization was described for determining several neurosteroids in both CSF and plasma samples. As described previously (19), the conversion of carbonyl groups in steroid molecules to carboxymethoximes, followed by pentafluorobenzyl esterification, not only provided characteristic ions $(M\text{-}181)^-$ for neurosteroid identification but also greatly enhanced sensitivity for quantitative determination. This GC/MS method along with the solid-phase extraction procedure provided a sensitive analytical procedure to allow the quantification of androsterone, dihydrotestosterone, testosterone, four stereoisomers of THP, and pregnenolone at low picogram/milliliter levels. We demonstrate in this paper that this method can be employed for the determination of these neurosteroids in CSF as well as plasma samples. The allopregnanolone and pregnenolone levels observed in this study were comparable with those reported using mass spectrometry (28, 29), but significantly lower than the reported values generated by radioimmunoassay (17, 30). Since the background peak eluted from the solid-phase extraction column obscured the detection of 5α,3α-THDOC and the overall reproducibility was poor for DHEA analysis, these steroids could not be evaluated in the present study. In conjunction with pharmacological intervention, the current method can be employed to investigate neurosteroid metabolism and its

TABLE 3

Neurosteroid Concentrations in Human and Monkey CSF

Neurosteroids	Human CSF (male, n = 5)	Monkey CSF (male, n = 6)
Androsterone	48 ± 17[a]	37 ± 9
Dihydrotestosterone	nd	nd
Testosterone	190 ± 37	90 ± 52
Allopregnanolone	52 ± 35	6 ± 2
Pregnanolone	nd	nd
Isopregnanolone	nd	12 ± 4
Pregnenolone	43 ± 17	7 ± 5

Note. As described under Materials and Methods, 1–2 ml CSF samples was analyzed after the addition of deuterium-labeled internal standards. Neurosteroids were identified according to their characteristic ions of $(M\text{-}181)^-$ and quantified against the known amount of internal standards.

[a] The values were expressed as pg/ml. nd, not detected.

TABLE 4

Neurosteroid Concentrations in Human and Rat Plasma Samples

Neurosteroids	Human plasma (male, n = 4)	Rat plasma (male, n = 8)
Androsterone	35,143 ± 4,292[a]	239 ± 78
Dihydrotestosterone	120 ± 47	nd
Testosterone	2,734 ± 419	2,637 ± 886
Epipregnanolone	nd	112 ± 29
Allopregnanolone	71 ± 20	116 ± 49
Pregnanolone	110 ± 37	nd
Isopregnanolone	111 ± 24	nd
Pregnenolone	4,175 ± 878	146 ± 78

Note. As described under Materials and Methods, 300 μl of plasma samples was analyzed after the addition of deuterium-labeled internal standards. Neurosteroids were identified according to their characteristic ions of $(M\text{-}181)^-$ and quantified against the known amount of internal standards. The concentration values of androsterone and pregnenolone determined using 20 μl of human plasma were similar to those shown in this table.

[a] The values were expressed as pg/ml. nd, not detected.

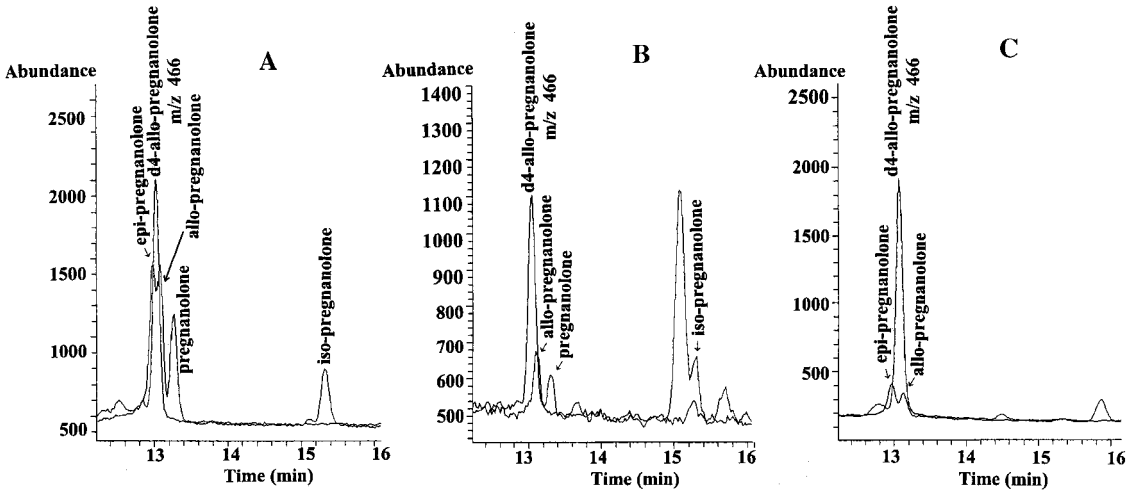

FIG. 6. Separation and detection of pregnanolone isomers in human and rat plasma. (A) GC/ECNCI/MS selected ion chromatograms obtained from a standard mixture containing 280 pg of d_4-allopregnanolone (*m/z* 466) and 200 pg each of pregnanolone and epi-, allo-, and isopregnanolone (*m/z* 462). (B) GC/ECNCI/MS selected ion chromatograms of pregnanolone isomers detected in human plasma spiked with 140 pg of d_4-allopregnanolone. Allopregnanolone (95.5 pg/ml), pregnanolone (150.1 pg/ml), and isopregnanolone (139.0 pg/ml) were detected. (C) GC/ECNCI/MS selected ion chromatograms of pregnanolone isomers detected in rat plasma spiked with 280 pg of d_4-allopregnanolone. Epipregnanolone (143.9 pg/ml) and allopregnanolone (88.9 pg/ml) were detected without pregnanolone and isopregnanolone.

physiological implications in various psychiatric disorders.

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REFERENCES

1. Baulieu, E. E. (1981) *in* Steroid Hormone Regulation of the Brain (Fuxe, K., Gustafsson, J. A., and Wetterberg, L., Eds.), pp. 3–14, Pergamon, Oxford, UK.
2. Baulieu, E. E. (1997) Neurosteroids: Of the nervous system, for the nervous system. *Rec. Prog. Horm. Res* **52,** 1–32.
3. Majewska, M. D. (1992) Neurosteroids: Endogenous bimodal modulators of the GABAA receptor. Mechanism of action and physiological significance. *Prog. Neurobiol.* **38,** 379–395.
4. Rupprecht, R. (1997) The neuropsychopharmacological potential of neuroactive steroids. *J. Psychiatr. Res.* **31,** 297–314.
5. Majewska, M. D., Harrison, N. L., Schwartz, R. D., Barker, J. L., and Paul, S. M. (1986) Steroid hormone metabolites are barbiturate-like modulators of GABA receptor. *Science* **232,** 1004–1007.
6. Gee, K. W., Brinton, R. E., Chang, W. C., and McEwen, B. S. (1987) $GABA_A$-dependent modulation of the Cl^- ionophore by steroids in rat brain. *Eur. J. Pharmacol.* **136,** 419–423.
7. Morrow, A. L., Suzdak, P. D., and Paul, S. M. (1987) Steroid hormone metabolites potentiate GABA receptor-mediated chloride ion flux with nanomolar potency. *Eur. J. Pharmacol.* **142,** 483–483.
8. Mendelson, W. B., Martin, J. V., Perlis, M., Wagner, R., Majeswska, M. D., and Paul, S. M. (1987) Sleep induction by an adrenal steroid in the rat. *Psychopharmacology* **93,** 226–229.
9. Kavaliers, M. (1988) Inhibitory influences of the adrenal steroid, 3 alpha, 5 alpha-tetrahydrodeoxycorticosterone [correction for tetrahydroxycorticosterone analgesia in mice] on aggression and defeat-induced. *Psychopharmacology* **95,** 488–492.
10. Bitran, D., Hilvers, R. J., and Kellogg, C. K. (1991) Anxiolytic Effect of 3α-hydroxy-5α[β]-pregnan-20-one: Endogenous metabolite of progesterone that are active at the $GABA_A$ receptor. *Brain Res.* **561,** 157–161.
11. Majeswska, M. D., Bluet-Pajot, M.-T., Robel, P., and Baulieu, E. E. (1989) Pregnenolone sulfate antagonizes barbiturate-induced hypnosis. *Pharmacol. Biochem. Behav.* **33,** 701–703.
12. Demirgoren, S., Majeswska, M. D., Spivak, C. E., and London, E. D. (1991) Receptor binding and electrophysiological effect of dehydroepiandrosterone sulfate, an antagonist of the GABAa receptor. *Neuroscience* **45,** 127–135.
13. Barbaccia, M. L., Roscetti, G., Trabucchi, M., Purdy, R. H., Mostallino, M. C., Perra, C., Concas, A., and Biggio, G. (1996) Isonizid-induced inhibition of GABAergic transmission enhances neurosteroid content in the rat brain. *Neuropharmacology* **35,** 1299–1305.
14. Uzunov, D. P., Cooper, T. B., Costa, E., and Guidotti, A. (1996) Fluoxetine-elicited changes in brain neurosteroid content measured by negative ion mass fragmentography. *Proc. Natl. Acad. Sci USA* **93,** 12599–12604.
15. Do-Rego, J. L., Mensah-Nyagan, A. G., Feuilloley, M., Ferrara, P., Pelletier, G., and Vaudry, H. (1998) The endozepine triakontatetraneuropeptide diazepame-binding inhibitor[17-50] stimulates neurosteroid biosynthesis in the frog hypothalamus. *Neuroscience* **83,** 555–570.
16. Makela, S. K., Ellis, G. (1988) Nonspecificity of a direct 17α-hydroxyprogesterone radioimmunoassay kit when used with samples from neonate, *Clin. Chem.* **34,** 2070–2075.
17. Purdy, R. H., Moore, P. H., Jr., Rao, P. N., Hagino, N., Yamaguchi, T., Schmidt, P., Rubinow, D. R., Morrow, A. L., and Paul, S. M. (1990) Radioimmunoassay of 3α-hydroxy-5α-pregnan-20-one in rat and human plasma. *Steroids* **55,** 290–296.

18. Hsu, F.-F., Wand, L., and Bier, D. M. (1994) Determination of cortisol in human plasma by gas chromatography/negative ion chemical ionization/mass spectrometry. *Anal. Biochem.* **216,** 401–405.
19. Hubbard, W. C., Bickel, C., and Schleimer, R. P. (1994) Simultaneous quantitation of endogenous levels of cortisone and cortisol in human nasal and bronchoalveolar lavage fluids and plasma via gas chromatography-negative ion chemical ionization mass spectrometry. *Anal. Biochem.* **221,** 109–117.
20. Legrand, C., Dousset, B., Tronel, H., Belleville, F., and Nabet, P. (1995) Measurement of plasma testosterone by gas chromatography–negative-ion mass spectrometry using pentafluoropropionic derivatives. *J. Chromatogr. B Biomed. Appl.* **663,** 187–192.
21. Kessler, M. J. (1982) Analysis of steroids from normal and tumor tissue by HPLC. *Clin. Chim. Acta* **125,** 21–30.
22. Esteban, N. V., and Yergey, A. L. (1990) Cortisol production rates measured by liquid chromatography/mass spectrometry. *Steroids* **55,** 152–158.
23. Ma, Y.-C., and Kim, H.-Y. (1997) Determination of steroids by liquid chromatography/mass spectrometry. *J. Am. Soc. Mass Spectrom.* **8,** 1010–1020.
24. Fredline, V. F., Taylor, P. J., Dodds, H. M., and Johnson, A. G. (1997) A reference method for the analysis of aldosterone in blood by high-performance liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry. *Anal Biochem* **252,** 308–313.
25. Fils-Aime, M. L, Eckardt, M. J., George, D. T., Brown, G. L., Mefford, I., and Linnoila, M. (1996) Early-onset alcoholics have lower cerebrospinal fluid 5-hydroxyindoleacetic acid levels than late-onset alcoholics. *Arch. Gen. Psychiatry* **53,** 211–216.
26. Zajicek, K. B., Higley, J. D., Suomi, S. J., and Linnoila, M. (1997) Rhesus macaques with high CSF 5-HIAA concentrations exhibit early sleep onset. *Psychiatr. Res.* **73,** 15–25.
27. Baulieu, B. B. (1998) Neurosteroids: A novel function of the brain. *Psychoneuroendocrinology* **23,** 963–987.
28. Romeo, E., Strohle, A., Spalletta, G., Michele, F., Hermann, B., Holsboer, F., Pasini, A., and Rupprecht, R. (1998) Effects of antidepressant treatment on neuroactive steroids in major depression. *Am. J. Psychiatr.* **155,** 910–913.
29. Uzunova, V., Sheline, Y., Davis, J. M., Rasmusson, A., Uzunov, D. P., Costa, E., and Guidotti, A. (1998) Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. *Proc. Natl. Acad. Sci USA* **95,** 3239–3244.
30. Barbaccia, M. L., Roscetti, G., Trabucchi, M., Mostallino, M. C., Concas, A., Purdy, R., and Biggio, G. (1996) Time-dependent changes in rat brain neuroactive steroid concentrations and GABAa receptor function after acute stress. *Neuroendocrinology* **63,** 166–172.